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Functional Genomics for Epithelial-Mesenchymal Transition in Breast Cancer

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To assess whether modulation of the PMC42-LA cell line by a library of shRNAmir constructs can cause tumorigenicity, the impact of a smaller boutique library is first being assessed, as proposed. This boutique library of 4,462 shRNA constructs targeting 1,860 markers and mediators of EMT, metastasis, migration, and BCSC has been transduced into the PMC42-LA cell line as a total pool and as ten smaller sub-pools. These were expanded in preparation for in vivo testing. The smaller sub-pools have greater representation of individual shRNAmir constructs. For any sub-pool containing shRNAmir constructs that enables tumorigenicity, this greater representation translates to a markedly larger inoculum of tumorigenic cells, which may be important in the establishment of a tumor. The presence of a small but significant proportion of colonies exhibiting markedly mesenchymal features has been observed in the transduced pools, validating the use of this boutique library pool to directly assess whether PMC42-LA tumors can be grown using pools containing a proportion of more mesenchymal cells as an inoculum prior to assessing the whole genome shRNAmir library. Various methodologies for the in vitro selection of transduced cells with mesenchymal features from these pools, supplementary to the initial aims, are under development.

**15. SUBJECT TERMS**
Key words or phrases identifying major concepts in the report: Epithelial Mesenchymal Transition (EMT), functional genomics, micrometastasis, macrometastasis, shRNA

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## Introduction

We hypothesize that epithelial – mesenchymal phenotypic attributes affect the capacity of single cells to establish a macroscopically detectable cancer mass, and thus play an etiological role in tumorigenicity, invasion, metastasis and recovery after seemingly effective chemotherapy of breast cancer cells. We will employ high throughput functional genomic screens using epithelial mesenchymal transition (EMT) -capable PMC42 and MDA-MB-468 human breast cancer cell lines to identify molecular factors controlling these processes, and test their relationship to the EMT process. This will increase our understanding of the role of EMT in breast cancer, provide novel reagents and tools for the study of breast cancer, and provide new leads for therapeutic targeting in breast cancer.

## Body

**Task 1: To identify gene products which may constitutively block the growth of PMC42 human breast cancer cells in SCID mice.**
These cells undergo EGF-induced EMT and show BCSC attributes, but do not grow in SCID mice when implanted orthotopically or introduced intra-cardially.

1a **(months 1-3)** Luciferase introduction into PMC42-LA.
**Deliverables:** Luciferase-tagged cells for tracking of metastasis in vivo.

Our initial efforts to construct a vector with a strong constitutive promoter expressing *luc2*, a modified form of luciferase that enables greater sensitivity, and with a drug-selectable marker compatible with the simultaneous use of both shRNAmir library constructs and promoter reporter constructs, were unsuccessful and caused considerable delay. As detection of orthotopic growth is not reliant upon the use of luciferase-tagged cells and detection of disseminated growth by other means is feasible for small scale experiments, and to avoid further delays, we decided to proceed with transduction of the PMC42-LA cell line with the boutique library without prior luciferase-tagging. Subsequently, an appropriate vector (pGL4.50[luc2/CMV/Hygro]) has become commercially available and will be used to luciferase-tag the PMC42-LA cell line prior to transduction with the whole genome shRNAmir library.

1b **(months 3-6)** Pre-titration of shRNAmir library to determine optimal multiplicity of infection.
**Deliverables:** Optimal MOI for PMC42-LA cells

A viral supernatant with a known viral titre has been re-titrated in the PMC42-LA cell line, enabling the viral dose required to give the optimal MOI to be determined. From the comparative titration it was determined that the PMC42-LA cell line requires 310-fold more virus as it is less efficiently transduced. This has been used for the successful transduction of these cells as below.

1c **(months 6-9)** Transduction of luciferase-tagged PMC42-LA with boutique shRNAmir libraries, followed by expansion and enrichment of transduced cells.
**Deliverables:** Stocks of luciferase-tagged PMC42-LA cells, transduced with a boutique library comprising 3,600 shRNA clones targeting 1,746 genes selected as markers and mediators of EMT, metastasis, migration, and BCSC.

A boutique library comprising 4,462 shRNAmir constructs targeting 1,860 markers and mediators of EMT, metastasis, migration, and BCSC has been transduced into the PMC42-LA cell line both as a total pool and as ten smaller sub-pools. These have been expanded in preparation for *in vivo* testing. The smaller sub-pools have greater representation of individual shRNAmir constructs. For any sub-pool containing shRNAmir constructs that enable tumorigenicity, this greater representation translates to a markedly larger inoculum of tumorigenic cells, which may be important in the establishment of a tumor in this model. The presence of a small but statistically significant proportion of colonies exhibiting markedly mesenchymal features has been observed in the transduced pools (Figure 1), validating the use of this boutique library pool to directly assess whether PMC42-LA tumors can be grown using pools containing a proportion of more mesenchymal cells as an inoculum prior to assessing the whole genome shRNAmir library.

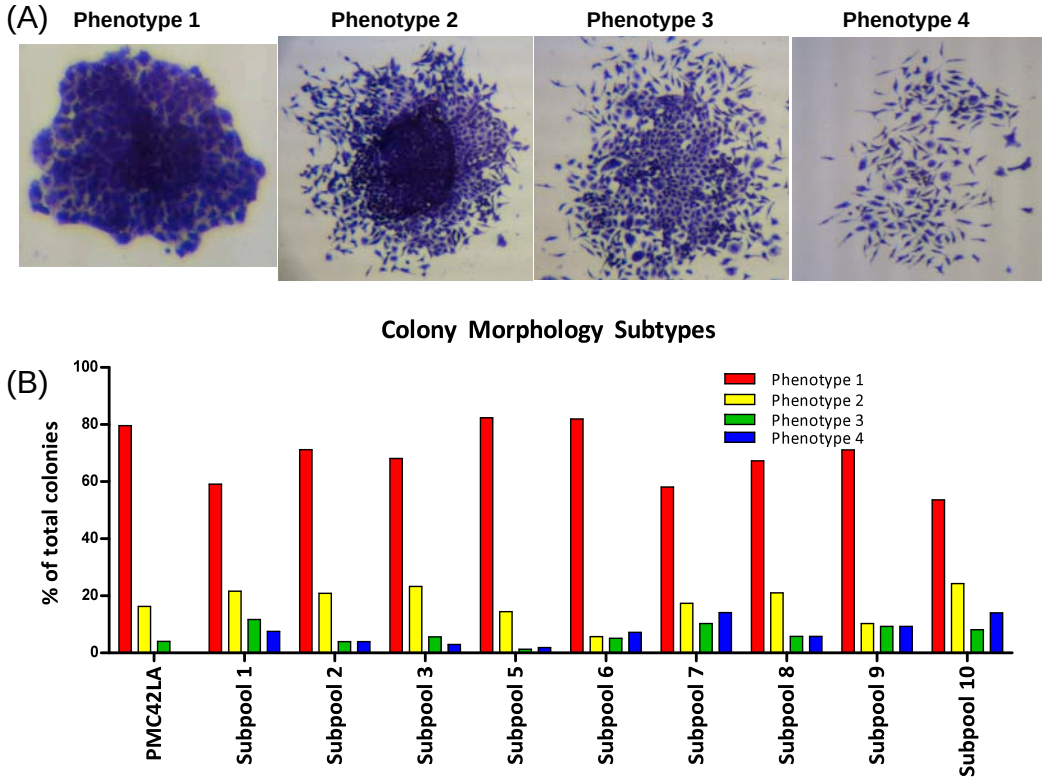


**Figure 1** Presence of mesenchymal colonies following transduction of PMC42-LA cells with shRNAmir library pools. Wild-type cells and the transduced subpools were plated at low density and the colonies categorised into four phenotypes (A). The relative number of each phenotype was determined (B).

1d **(months 9-12)** Orthotopic and Disseminated growth of boutique shRNAmir transduced PMC42-LA cells.
**Deliverables:** Tumors in which a block in tumorigenicity has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Transduced pools have been expanded for inoculation, but were not inoculated by the end of this reporting period. They have since been inoculated intracardially, to allow for disseminated growth, and into the orthotopic site.

1e **(months 12-18)** Transduction of luciferase-tagged PMC42-LA with whole genome shRNAmir libraries, followed by expansion and enrichment of transduced cells.
**Deliverables:** Stocks of luciferase-tagged PMC42-LA cells, transduced with ~ 100,000 whole genome shRNA clones.

Not covered in year 1

1f **(months 9-12)** Orthotopic **or** Disseminated growth of whole genome shRNAmir transduced PMC42-LA cells.
**Deliverables:** Tumors in which a block in tumorigenicity has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

As above, this has been delayed and will now be performed in year 2.

**Task 2: To identify gene products which may constitutively block the spontaneous capacity of MDAMB-468 human breast cancer cells to form metastases in SCID mice.**
These cells undergo EMT at the xenograft edge and liberate CTCs, but form micrometastases at low frequency. They do not form overt lesions when inoculated intracardially. After genome-wide shRNA knockdown they will be screened for disseminated growth following intra-cardiac seeding, and distant metastasis following orthotopic inoculation.

2a **(months 1-3)** Luciferase introduction into MDA-MB-468.
**Deliverables:** Luciferase-tagged MDA-MB-468 cells for tracking of metastasis in vivo.

As above for PMC42, our initial efforts to construct a vector with a strong constitutive promoter expressing *luc2*, a modified form of luciferase that enables greater sensitivity, and with a drug-selectable marker compatible with the simultaneous use of both shRNAmir library constructs and promoter reporter constructs were unsuccessful. Subsequently, an appropriate vector (pGL4.50[luc2/CMV/Hygro]) has become commercially available and will be used to luciferase-tag the MDA-MB-468 cell line prior to transduction with the shRNAmir libraries.

2b **(months 3-6)** Pre-titration of shRNAmir library to determine optimal multiplicity of infection.
**Deliverables:** Optimal MOI for MDA-MB-468 cells

Awaiting *luc2*-tagging of the MDA-MB-468 cells. Unlike the PMC42 cells, where the screen relies on growth at the primary site such that Luc-tagging is not essential, the MDA-MB468 screen is for metastatic growth and thus does require the luc-tagged cells.

2c **(months 6-9)** Transduction of luciferase-tagged MDA-MB-468 with boutique shRNAmir libraries, followed by expansion and enrichment of transduced cells.
**Deliverables:** Stocks of luciferase-tagged MDA-MB-468 cells, transduced with a boutique library comprising 3,600 shRNA clones targeting 1,746 genes selected as markers and mediators of EMT, metastasis, migration, and BCSC.

Tasks 2c and 2d were added to the SOW in error, as they are neither set out in the narrative nor covered in the budget. However, we do feel that it would be advantageous to have the boutique library arm included and we will look to include this within the current budget and with appropriate modification of the mouse numbers in the animal ethics.

2d **(months 9-12)** Disseminated growth of boutique shRNAmir transduced MDA-MB-468 cells.
**Deliverables:** Tumors in which a block in macrometastasis has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Same as remarks regarding task 2c.

2e **(months 12-18)** Transduction of luciferase-tagged MDA-MB-468 with whole genome shRNAmir libraries, followed by expansion and enrichment of transduced cells.
**Deliverables:** Stocks of luciferase-tagged MDA-MB-468 cells, transduced with ~ 100,000 whole genome shRNA clones.

Not covered in year 1

2f **(months 19-24)** Disseminated growth of whole genome shRNAmir transduced MDA-MB-468 cells.
**Deliverables:** Tumors in which a block in macrometastasis has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Not covered in year 1

**Task 3: To identify and characterise novel gene products elucidated in Aims 1 and 2 that enact a switch between epithelial and mesenchymal states.**
Candidate shRNAs identified in Aims 1 and 2 will be assembled individually or assembled into a smaller, dedicated boutique screen and tested for their ability to influence EMT or MET. Novel candidates confirmed to play a role in EMT will be tested fully with *in vitro* and *in vivo* analysis.

3a **(months 1-12)** Transduction of various human breast cancer cell lines with epithelial (E-Cad-RFP) or mesenchymal (Vimentin-RFP) reporter constructs, and validation with known EMT perturbations.
**Deliverables:** Cells lines which faithfully report EMT and MET shifts.

Direct adaptation of the reporter constructs to our cell lines has not been automatic and improved reporter constructs are being developed in conjunction with AI Williams, including the substitution of a blue-FP for RFP in one of the vectors, which will improve the functionality of the reporter system by allowing for simultaneous measurements of epithelial and mesenchymal

promoter states in the presence of the GFP-encoding GIPZ shRNA constructs. These are not required until the start of year 3, and will be further refined towards that date. Subsequent to the current reporting period, four cell lines have been successfully transfected with a Vimentin-RFP reporter construct. Two rounds of FACS sorting have been applied to ensure that the drug resistant transfected cell populations contain only cells with a demonstrable capacity for reporter expression, while precluding any cells that may have had constitutive reporter expression as a consequence of the integration site (Figure 2).

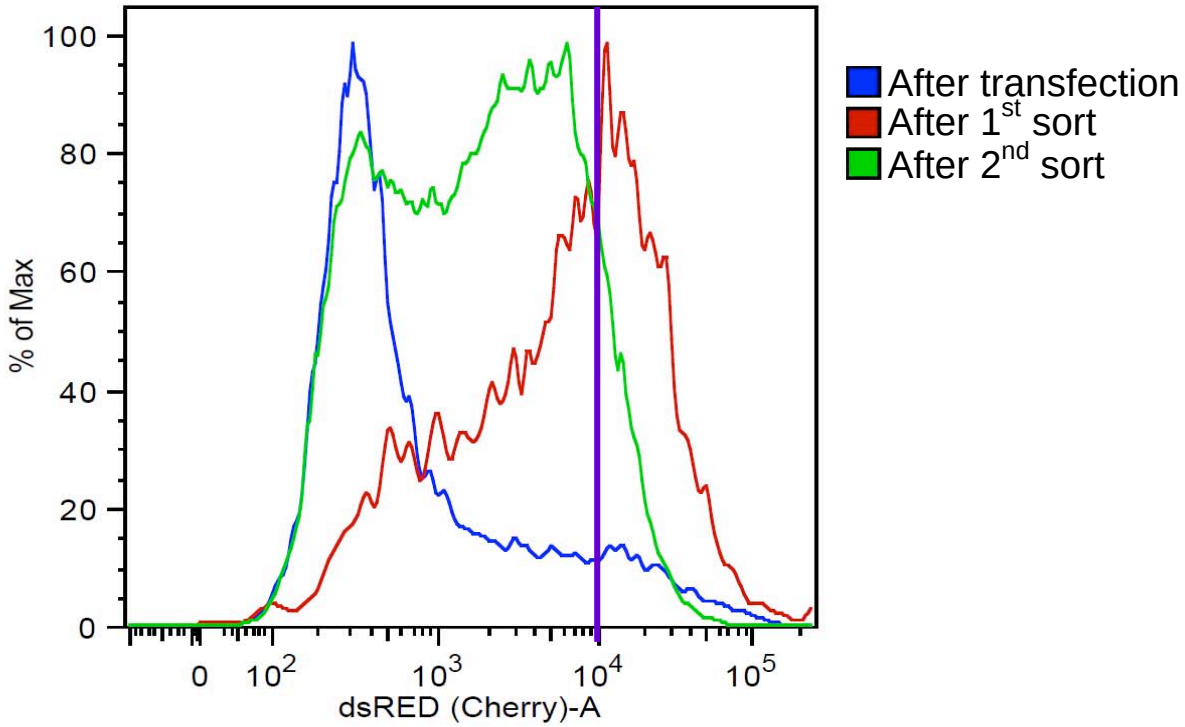


**Figure 2** Representative cell line demonstrating FACS selection procedure. MDA-MB-468 cells were transfected with a Vimentin-RFP reporter construct and following drug selection were selected by FACS for high (cut-off indicated by purple line) expression, ensuring that the reporter construct had integrated in such a way as to be able to be expressed in the selected cells. These cells were cultured prior to selection by FACS for cells with low expression, removing from the population any cells in which integration had resulted in constitutive expression. Assessment of the range of reporter expression levels following culture of this double sorted population revealed a broad dynamic range that included a proportion of cells that lacked detectable promoter expression, in line with expectations for this cell line.

3b **(months 18-24)** Deep sequencing of assembled DNA barcodes / hairpins pooled from colonies arising in both boutique and whole genome screens in Tasks 1 and 2**.**
**Deliverables:** Sequence identification of shRNAs present in colonies escaping repression of tumorigenesis and/or macrometastasis from Aims 1 and 2 respectively.

Not covered in year 1

3c **(months 24-27)** Screen boutique shRNA library with FACS-based, in-cell EMT/MET reporter assays
**Deliverables:** Identification of shRNA candidates with EMT perturbation potential.

Not covered in year 1

3d **(months 24-36)** Designation of EMT/MET/BCSC and non EMT/MET/BCSC candidates.
**Deliverables:** Prioritization of EMT/MET/BCSC for this study, dissemination of non-; prioritization of EMT/MET/BCSC to the research community.

Not covered in year 1

3e **(months 27-36)** In vitro analysis of up to 10 EMT/MET perturbational candidates.
**Deliverables:** Characterization of the in vitro proliferative, migratory, and invasion-regulating potential of candidates, analysis of EMT perturbational mechanism, analysis of relationship to BCSC phenotype.

Not covered in year 1

3f **(months 30-36)** In vivo analysis of up to 3 EMT/MET perturbational candidates for effects on tumorigenic potential of PMC42-LA and macrometastatic potential of MDA-MB-468; analysis of molecular consequences and morphologic effects in vivo.
**Deliverables:** Characterization of the in vivo tumorigenic and macrometastatic potential of candidates, analysis of EMT perturbational mechanism, analysis of relationship to BCSC phenotype.

Not covered in year 1

3g **(months 24-36)** Manuscript preparation and submission, grant preparation.
**Deliverables:** Submitted grant applications for further work, dissemination of findings through conferences and publications.

Not covered in year 1

**Key Research Accomplishments**

- The viral transduction efficiency of the PMC42-LA cell line has been determined, enabling the optimal MOI to be used for the transduction of the shRNAmir library pools.

- A boutique library comprising 4,462 shRNAmir constructs targeting 1,860 markers and mediators of EMT, metastasis, migration, and BCSC has been transduced into the PMC42-LA cell line both as a total pool and as ten smaller sub-pools.

- All PMC42-LA cell line pools have been expanded in preparation for *in vivo* testing.

- We have validated the use of this boutique library to directly assess whether PMC42-LA tumors can be grown using pools containing a proportion of more mesenchymal cells as an inoculum prior to assessing the whole genome shRNAmir library, through the presence of a small but significant proportion of colonies exhibiting markedly mesenchymal features in the transduced pools.

**Reportable Outcomes**

***PRIMARY RESEARCH ARTICLES***

Blick, T., Hugo, H., Widodo, E., Waltham, M., Pinto, C., Mani, S.A., Weinberg, R.A., Neve, R.M., Lenburg, M.E., THOMPSON, E.W. Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44hi/CD24lo/- stem cell phenotype in human breast cancer. J. Mammary Gland Biol. Neoplasia. Special issue EMT in Mammary Development and Breast Cancer Progression 15: 235-252 (2010) DOI:10.1007/s10911-010-9175-z

Hugo, H.J., Kokkinos, M.I., Blick, T., Ackland, M.L., THOMPSON, E.W., Newgreen, D.F. Defining the E-cadherin repressor interactome in epithelial-mesenchymal transition – The PMC42 model as a case study. Cells, Tissues, Organs November 2, 2010 [Epub ahead of print] DOI: 10.1159/000320174

***REVIEWS***

Bonnomet, A., Brysse, A., Tachtsidis, A., Waltham, M., THOMPSON, E.W., Polette, M., Gilles, C. Epithelial-to-mesenchymal transitions and circulating tumor cells. J. Mam. Gland Biol. Neoplasia. Special issue EMT in Mammary Development and Breast Cancer Progression 15: 261-273 (2010). DOI:10.1007/s10911-010-9174-0

**BOOK CHAPTERS**

Soon, L, Tachtsidis, A, Fok, S, Williams, ED, Newgreen, DF, THOMPSON, EW. The continuum of epithelial mesenchymal transition – implication of hybrid states for migration and survival in development and cancer. In Cancer Metastasis: Biologic Basis and Therapeutics. DR Welch and DC Lyden, eds. Cambridge Press. In press (02/10)

***OTHER PUBLICATIONS***

Ford, H., THOMPSON, E.W. Guest Editors. Special Topic Issue: Epithelial Mesenchymal Transitions in Mammary Development and Breast Cancer Progression. J. Mammary Gland Biology & Neoplasia 2010. Preface - Ford H.L., THOMPSON E.W. Mammary gland studies as important contributors to the cause of epithelial mesenchymal plasticity in malignancy. J. Mammary Gland Biol. Neoplasia 15: 113-115 (2010)

***CONFERENCES PRESENTATIONS***

2010 19th Annual Meeting of the Japanese Association of Metastasis Research, Kanazawa, Japan; June 16-17. International Symposium Co-chair

AACR Special Conference on EMT and Cancer Progression and Treatment, Arlington, VA; Feb.28-March2. Insights into the Human Breast Cancer EMT from Established Cell Lines

***SEMINAR PRESENTATIONS***

2010 Whitehead Institute, MIT, Boston, USA (host Prof. R. Weinberg); Sept.17. Epithelial mesenchymal plasticity in PMC42 human breast cancer cells.

Princeton University, Dept. of Molecular Biology (host Dr. Y. Kang); Sept.16. The yin yang of epithelial mesenchymal plasticity in carcinoma progression.

Kanazawa University Cancer Research Institute, Kanazawa University, Kanazawa, Japan; June 22. Insights into the human breast cancer EMT from established cell lines

Diamantina Institute for Cancer, Immunology and Metabolic Medicine (Host Prof Tom Gonda), University of Queensland, Princess Alexandria Hospital, QLD; Aug. 27. Epithelial Mesenchymal Plasticity in Breast Cancer

**CONCLUSION**

This research project was designed to generate analysable results in the latter stages of the project. At the current stage it is too early to draw any conclusions.